IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appln No. : 10/593,538
Applicants : Ippei SAKIMOTO et al.
Filed : September 19, 2006
For : RADIOSENSITIZER
Art Unit : 4131
Examiner : SCARLETT GOON

DECLARATION UNDER 37 C.F.R. 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231
Sir:

I, Keisuke Ohta, declare as follows.

I am a co-applicant of the above-identified application.

I graduated from Tokyo University of Science in 1993, and have worked in Research and Development Division of TOYO SUISAN KAISHA, LTD. from 1993 to now. I have been engaged in development of medicine in this company. I received Ph.D from Tokyo University of Science in 2001 during my tenure in this company.

I have conducted the following experiments.

Experiments:

Experiment 1:

Experiment 1 demonstrates that a method of the present invention is effective in the treatment of prostate adenocarcinoma.

Method:

Experiment 1 was carried out in a manner similar to the Experimental Example 1-1 (the present specification, page 16, line 17 to page 17, line 23) as follows:

Nude mice were assigned to the following four groups (4 mice per group):

(1) control group;

(2) single use group of the present radiosensitizer administration;

(3) single use group of irradiation; and

(4) combined use group of irradiation and the present radiosensitizer administration.

2.0×10^6 human prostate adenocarcinoma cells (DU145 cells) were suspended in

PBS(-), and the suspension was transplanted subcutaneously into a right thigh of each mouse. When tumor volume became about 50 mm^3, each mouse was subjected to treatment according to each group.

As a radiosensitizer, 3-O-(6-deoxy-6-sulfo-α-D-glucopyranosyl)-1-O-stearoyl-glycerol sodium salt (hereinafter, also referred to as "α-SQMG C18:0") was used. Regarding the single use group of irradiation, X-ray was irradiated two times (at the time of treatment initiation (day 0) and on day 4 after the treatment initiation) at a dose of 4 Gy. Regarding the single use group of α-SQMG C18:0 administration, the radiosensitizer was intraperitoneally administered at a concentration of 1 mg/kg once a day at a total of 5 times from the time of treatment initiation (day 0) to day 4. Regarding the combined use group, each mouse of the group was treated with both of irradiation (two times) and α-SQMG C18:0 administration (five times). Regarding the control group, neither irradiation nor α-SQMG C18:0 administration was performed.

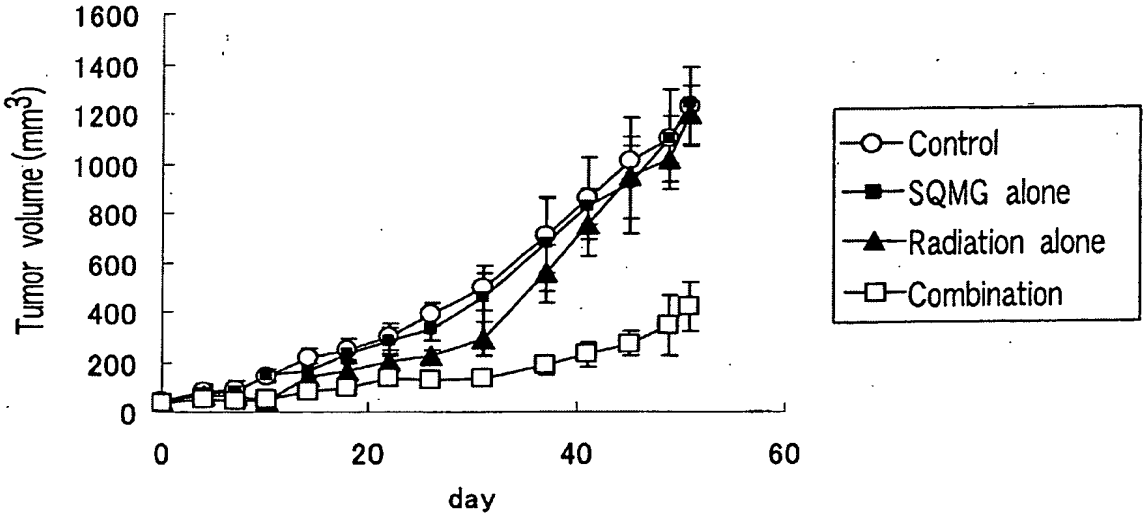
Thereafter, a short diameter and a long diameter of a tumor were measured with a micrometer caliper. Tumor volume was calculated by the following equation.

Equation for calculating tumor volume:

tumor volume (mm^3) = (short diameter)^2 × (long diameter) × 0.5

Results:

The results obtained are shown in the following graph.



The results show that a method of the present invention (combined therapy) is effective in prostate adenocarcinoma derived from adenocarcinoma, and that a method of the present invention (combined therapy) has a synergistic effect exceeding an expected additive effect of irradiation and α-SQMG C18:0 administration.

Experiment 2:

Experiment 2 demonstrates that a method of the present invention is effective in the treatment of colorectal adenocarcinoma.

Method:

Experiment 2 was carried out in a manner similar to the Experimental Example 1-1 (the present specification, page 16, line 17 to page 17, line 23) as follows:

Nude mice were assigned to the following four groups (4 mice per group):

(1) control group;

(2) single use group of the present radiosensitizer administration;

(3) single use group of irradiation; and

(4) combined use group of irradiation and the present radiosensitizer administration.

2.0×10^6 human colorectal adenocarcinoma cells (SW480 cells) were suspended in PBS(-), and the suspension was transplanted subcutaneously into a right thigh of each mouse. When tumor volume became about 50 mm^3, each mouse was subjected to treatment according to each group.

As a radiosensitizer, 3-O-(6-deoxy-6-sulfo-α-D-glucopyranosyl)-1-O-stearoyl-glycerol sodium salt (hereinafter, also referred to as "α-SQMG C18:0") was used. Regarding the single use group of irradiation, X-ray was irradiated two times (at the time of treatment initiation (day 0) and on day 4 after the treatment initiation) at a dose of 4 Gy. Regarding the single use group of α-SQMG C18:0 administration, the radiosensitizer was intraperitoneally administered at a concentration of 1 mg/kg once a day at a total of 5 times from the time of treatment initiation (day 0) to day 4. Regarding the combined use group, each mouse of the group was treated with both of irradiation (two times) and α-SQMG C18:0 administration (five times). Regarding the control group, neither irradiation nor α-SQMG C18:0 administration was performed.

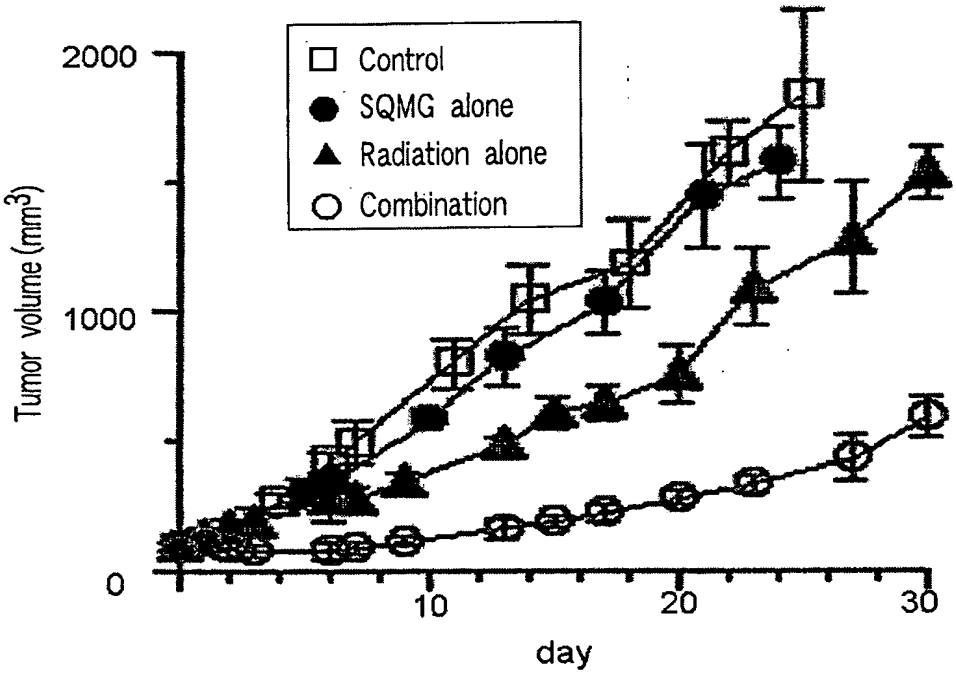
Thereafter, a short diameter and long diameter of a tumor were measured with a micrometer caliper. Tumor volume was calculated by the following equation.

Equation for calculating tumor volume:

tumor volume (mm^3) = (short diameter)^2 × (long diameter) × 0.5

Results:

The results obtained are shown in the following graph.



The results show that a method of the present invention (combined therapy) is effective in colorectal adenocarcinoma derived from adenocarcinoma, and that a method of the present invention (combined therapy) has a synergistic effect exceeding an expected additive effect of irradiation and α-SQMG C18:0 administration.

Experiment 3:

Experiment 3 demonstrates that a radiosensitizer of the present invention has a sufficient therapeutic effect even in combination with a low irradiation dose.

Method:

Experiment 3 was carried out in a manner similar to the Experimental Example 1-1 (the present specification, page 16, line 17 to page 17, line 23) as follows:

Nude mice were assigned to the following six groups (4 mice per group):

(1) control group;

(2) single use group of the present radiosensitizer administration;

(3) single use group of irradiation (2Gy);

(4) single use group of irradiation (6Gy);

(5) combined use group of irradiation (2Gy) and the present radiosensitizer administration; and

(6) combined use group of irradiation (6Gy) and the present radiosensitizer administration.

2.0×10^6 human colorectal adenocarcinoma cells (SW480 cells) were suspended in PBS(-), and the suspension was transplanted subcutaneously into a right thigh of each mouse. When tumor volume became about 200 mm^3, each mouse was subjected to treatment according to each group.

As a radiosensitizer, 3-O-(6-deoxy-6-sulfo-α-D-glucopyranosyl)-1-O-stearoyl-glycerol sodium salt (hereinafter, also referred to as "α-SQMG C18:0") was used. Regarding the single use group of irradiation, X-ray was irradiated two times (at the time of treatment initiation (day 0) and on day 4 after the treatment initiation) at a dose of 2 Gy or 6 Gy. Regarding the single use group of α-SQMG C18:0 administration, the radiosensitizer was administered into a caudal vein at a concentration of 2 mg/kg once a day at a total of 5 times from the time of treatment initiation (day 0) to day 4. Regarding the combined use group, each mouse of the group was treated with both of irradiation (two times) and α-SQMG C18:0 administration (five times). Regarding the control group, neither irradiation nor α-SQMG C18:0 administration was performed.

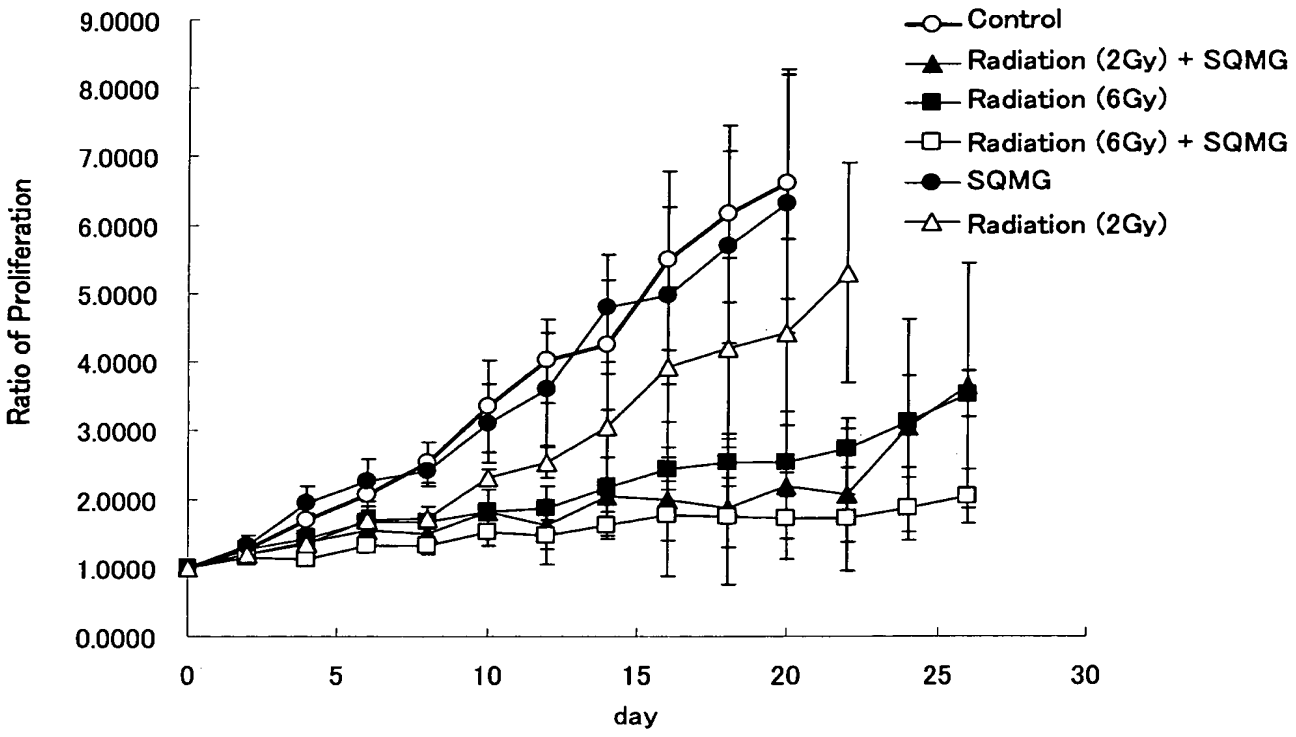
Thereafter, a short diameter, long diameter and height of a tumor were measured with a micrometer caliper. Tumor volume was calculated by the following equation, and a ratio of the obtained tumor volume value to the initial tumor volume (200 mm^3) at the time of day 0 was calculated.

Equation for calculating tumor volume:

tumor volume (mm^3) = (short diameter) × (long diameter) × (height) × π ÷ 6

Results:

The results obtained are shown in the following graph.



The results show that the combined therapy of irradiation at a dose of 2Gy and α-SQMG C18:0 administration (-▲- in the graph) has a high anti-tumor effect at the same level as both the case of irradiation at a dose of 6Gy (-■- in the graph) and the case of the combined therapy of irradiation at a dose of 6Gy and α-SQMG C18:0 administration (-□- in the graph). From the results, it is concluded that a method of the present invention can reduce a dose of radiation.

I, the undersigned, declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Cord and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: Aug. 6. 2008

Keisuke Ohta

Keisuke Ohta

Exhibit 1

<< Cell Detail Data >>

JCRB No.	JCRB0260
Cell Name	SAS
Profile	
Animal	human
Species	Homo sapiens
Sex	
Age	
Tissue	tongue
Case History	poorly defferentiated squamous cell carcinoma of tongue
Metastasis	
Genetics	hypertriploid
Lifespan	infinite
Morphology	epithelial-like
Characteristics	tongue, squamous carcinoma. cytokeratin-positive, alkaline phosphatase-positive. transplantable in nude mice.
Classification	
Establisher	Takahashi,K.
Depositor	Sato, K.
Medium	45% Dulbecco's modified Eagle's medium with 45% Ham's F12 medium and 10% fetal calf serum
Passage Method	Cells are treated with 0.02% EDTA and 0.1% trypsin.
Passage Cell No.	10^5 cells/60mm dish

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Exhibit 2

TKG 0259 :: TE-8

ID:
TKG 0259
Cell name:
TE-8
Animal:
Human.
Sex:
Male.
Scientific name:
Homo sapiens.
Tissue:
Cancer lesion of esophagus.
Passage method:
0.25% trpsin.
Life span:
Infinite.
Morphology:
Epithelial-like.
Medium:
RPMI-1640 + 10% FBS.
Characteristics:
Moderately differntiated squamous carcinoma (esophagus), transplantable to nude mouse. HLA-A 2402/2601, HLA-B B7/B61, HLA-C CW7/
Established by:
Sekine, Y. (Tohoku Univ.).
References:
Atlas of Human Tumor Cell Lines, 269-282, 1994. Academic Press. J. Cancer Res. Clin. Oncol., 119, 441-449, 1993. Cancer, 95, 737-743, 2002.
Deposited by:
Nishihira, T. (2nd Dept. Surgery, Tohoku Univ.)

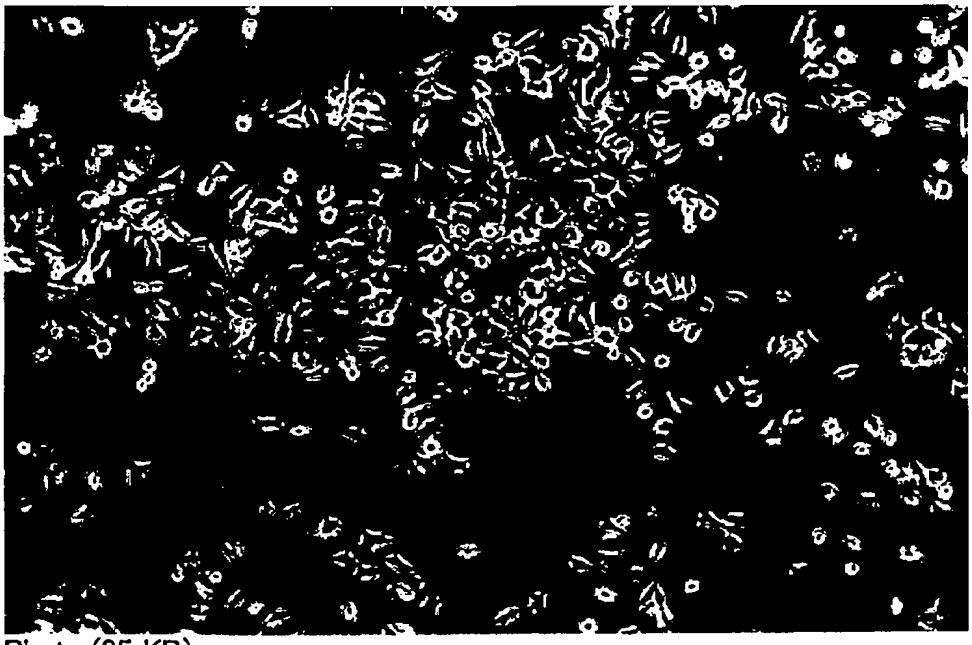

Photo (95 KB)

1: Cancer Lett. 2008 May 14. [Epub ahead of print]

ELSEVIER FULL-TEXT ARTICLE

2'-Nitroflavone induces cell cycle arrest and apoptosis in HeLa human cervical carcinoma cells.

Cárdenas MG, Blank VC, Marder M, Roguin LP.

Instituto de Química y Fisicoquímica Biológicas (UBA-CONICET), Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956 - 1113 Buenos Aires - Argentina.

The mechanism of antitumor action of a synthetic nitroflavone derivative, 2'-nitroflavone, was evaluated in vitro in HeLa human cervix adenocarcinoma cells. We showed that the nitroflavone derivative slowed down the cell cycle at the S phase and increase the population of cells at the G(2)/M phase after 24h of incubation. The treatment with 2'-nitroflavone also induced an apoptotic response, characterized by an increase of the sub-G1 fraction of cells, by cells with chromatin condensation and membrane blebbing, by a typical ladder of DNA fragmentation and by detection of apoptotic cells stained with Annexin V. The observed apoptosis was regulated by caspase-8 and -9, both contributing to the activation of the effector caspase-3. In addition, inhibitors of caspase-8 or -9 partially protected HeLa cells from 2'-nitroflavone-induced cell death. We also found that 2'-nitroflavone did not affect the total amount of Bax and Bcl-2 proteins, although a translocation of Bax from cytosol to mitochondria was evident after 6h of exposure. Furthermore, 2'-nitroflavone decreased the expression of the anti-apoptotic Bcl-X(L) protein, induced the release of cytochrome C to cytosol and increased the levels of Fas and Fas-L. Our results indicated that both death receptor and mitochondria-dependent pathways are involved in the apoptotic cell death triggered by 2'-nitroflavone and suggest that this derivative could be a potentially useful agent for the treatment of certain malignancies.

PMID: 18485587 [PubMed - as supplied by publisher]

Exhibit 4

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Cell Biology

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Price: $244.00

Designations: HeLa

Depositors: WF Scherer

Biosafety Level: 2

Shipped: frozen

Medium & Serum: See Propagation

Growth Properties: adherent

Organism: Homo sapiens (human)

Morphology: epithelial

PHOTO

Source: **Organ:** cervix
Disease: adenocarcinoma
Cell Type: epithelial

Cellular Products: keratin
Lysophosphatidylcholine (lyso-PC) induces AP-1 activity and c-jun N-terminal kinase activity (JNK1) by a pr kinase C-independent pathway [26623]

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Related Cell Culture P

Applications: transfection host([21491] technology from amaxa
Roche FuGENE® Transfection Reagents)
screening for Escherichia coli strains with invasive potential [21447] [21491]

Virus Susceptibility: Human adenovirus 3
Encephalomyocarditis virus
Poliovirus 1
Poliovirus 2
Human poliovirus 3

Reverse Transcript: negative

DNA Profile (STR): Amelogenin: X
CSF1PO: 9,10
D13S317: 12,13.3
D16S539: 9,10
D5S818: 11,12
D7S820: 8,12
THO1: 7
TPOX: 8,12
vWA: 16,18

Cytogenetic Analysis: Modal number = 82; range = 70 to 164.
There is a small telocentric chromosome in 98% of the cells. 100% aneuploidy in 1385 cells examined. Fou HeLa marker chromosomes have been reported in the literature.HeLa Marker Chromosomes: One copy of copy of M2, four-five copies of M3, and two copies of M4 as revealed by G-banding patterns. M1 is a re long arm and centromere of chromosome 1 and the long arm of chromosome 3. M2 is a combination of sl

Exhibit 5

<< Cell Detail Data >>

JCRB No.	JCRB0076
Cell Name	A549
Profile	Human adenocarcinoma cell line derived from lung cancer.
Animal	human
Species	Homo sapiens
Sex	M
Age	58 year-old
Tissue	lung
Case History	lung cancer
Metastasis	
Genetics	adenocarcinoma
Lifespan	infinite
Morphology	epithelial-like
Characteristics	Y chromosome invisible but Y specific DNA was detected.
Classification	tumor
Establisher	Giard,D.
Depositor	Akiyama,M.
Medium	Eagle's minimal essential medium with non essential amino acids and 10% fetal calf serum
Passage Method	Cells are treated with 0.02 % EDTA and 0.25 % trypsin.
Passage Cell No.	2x10^4 cells/sq.cm.

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Exhibit 6

1: J Natl Cancer Inst. 2007 Mar 7;99(5):376-85.

A prostate-specific antigen-activated channel-forming toxin as therapy for prostatic disease.

Williams SA, **Merchant RF**, **Garrett-Mayer E**, **Isaacs JT**, **Buckley JT**, **Denmeade SR**.

Department of Oncology, The Johns Hopkins University School of Medicine, Baltimore, MD 21231, USA.

BACKGROUND: Most men will develop prostatic abnormalities, such as benign prostatic hyperplasia (BPH) or prostate cancer, as they age. Prostate-specific antigen (PSA) is a serine protease that is secreted at high levels by the normal and diseased prostate. Therapies that are activated by PSA may prove effective in treating prostatic malignancies. METHODS: We modified proaerolysin (PA), the inactive precursor of a bacterial cytolytic pore-forming protein, to produce a PSA-activated protoxin (PRX302). The viability of the prostate adenocarcinoma cell lines LNCaP, PC-3, CWR22H, and DU145 and the bladder cancer cell line TSU after treatment with PA or PRX302 in the presence or absence of purified PSA was assayed. Mice carrying xenograft tumors derived from LNCaP, CWR22H, or TSU cells were treated with intratumoral injection of PA or PRX302, and tumor size was monitored. To test the safety of PRX302, we administered it into the PSA-secreting prostate glands of cynomolgus monkeys. All statistical tests were two-sided. RESULTS: Native PA was highly toxic in vitro but had no tumor-specific effects in vitro or in vivo. Picomolar concentrations of PRX302 led to PSA-dependent decreases in cell viability in vitro (PRX302 versus PRX302 + PSA: DU145 cells, mean viability = 78.7% versus mean = 1.6%, difference = 77.1%, 95% confidence interval [CI] = 70.6% to 86.1%; P<.001; TSU cells, mean = 100.2% versus mean = 1.4%, difference = 98.8%, 95% CI = 96.4% to 104.0%; P<.001). Single intratumoral injections of PRX302 produced substantial and often complete regression of PSA-secreting human prostate cancer xenografts (5 microg dose, complete regression in 6 of 26 mice bearing LNCap or CWR22H xenografts [23%]; 10 microg dose, complete regression in 10 of 26 mice [38.5%]) but not PSA-null bladder cancer xenografts. The prostates of cynomolgus monkeys injected with a single dose of PRX302 displayed extensive but organ-confined damage, with no toxicity to neighboring organs or general morbidity. CONCLUSIONS: Our observations demonstrate the potential safe and effective intraprostatic application of this engineered protoxin.

PMID: 17341729 [PubMed - indexed for MEDLINE]

Exhibit 7

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Cell Biology

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Biosafety Level:	1	**Shipped:**	frozen
Medium & Serum:	See Propagation	**Growth Properties:**	adherent
Organism:	Homo sapiens (human)	**Morphology:**	epithelial
			PHOTO
Source:	**Organ:** colon **Tumor Stage:** Dukes' type B **Disease:** colorectal adenocarcinoma		
Cellular Products:	carcinoembryonic antigen (CEA) 0.7 ng/10 exp6 cells/10 days; keratin; transforming growth factor beta		
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			Related Cell Culture P
Applications:	transfection host(technology from amaxa Roche FuGENE® Transfection Reagents)		
Receptors:	epidermal growth factor (EGF)		
Virus Susceptibility:	Human immunodeficiency virus 1		
Tumorigenic:	Y		
Reverse Transcript:	negative		
Antigen Expression:	HLA A2, B8, B17; blood type A; Rh+		
DNA Profile (STR):	Amelogenin: X CSF1PO: 13,14 D13S317: 12 D16S539: 13 D5S818: 13 D7S820: 8 THO1: 8 TPOX: 11 vWA: 16		
Cytogenetic Analysis:	The stemline chromosome number is hypotriploid and 11-12 marker chromosomes were common. Botl minutes and dicentrics were observed in 8% of each metaphase examined.		
Isoenzymes:	ES-D, 1 G6PD, B PEP-D, 1		